Pharmaceutical compositions having an effect on the proliferation of NK cells and a method using the same

The invention relates to pharmaceutical compositions having an effect on the proliferation of NK cells, to a method for specifically stimulating the proliferation of NK cells and to the use of same in the manufacture of a drug for the antitumoral prevention, palliation, and therapy of e.g., melanoma, hepatocarcinoma or lung adenocarcinoma and for anti-microbial prevention, palliation and therapy.

Some mechanisms of cytotoxicity of NK cells are known for a long time.

NK cells express CD16 molecule, which is a low affinity receptor for the Fc portion of IgG molecules. Thus NK cells recognize and kill antibody coated targets through recognition of the Fc portion of antibodies, that specifically recognize structures on the target cells.

NK cells also express so called Killer Inhibitory Receptors (KIR), which specifically recognize MHC class I molecule and inhibit the activation of cytolytic pathway in NK cells. Thus MHC class I positive targets are protected to a certain level from NK cell lysis.

Nevertheless, some targets, that do not express MHC class I positive targets are not killed by NK cells. This suggested that an active mechanism, distinct of CD16 or KIR molecule, can activate NK cells.

Several NK specific receptors have been identified that play an important role in the activation of NK cells.

Thus, NKp46 has been disclosed as active receptor responsible for triggering the natural cytotoxicity. More recently, other triggering receptor involved in NK cell mediated recognition and killing of target cells have also been disclosed. Moretta et al have thus disclosed a receptor of about 30 kD on SDS PAGE, designated NKp30 (US patent application s.n.10/036 444 divisional of US patent application s.n. 09/440 514).

Antibodies specific to these receptors, when coated to Fc receptor positive cells by their Fc moieties, trigger NK cell recognition and cytotoxicity in tests known as redirected killing assays.

It has been demonstrated that a lot of NK sensitive target are killed via one of these receptors, as Fab'2 or IgM specific for NkP46 or NKp30 abrogate most of the killing capacity of NK cells towards sensitive cells. This implies that specific ligands are present on sensitive cells for NKp46 and/or NKp30, though the molecular structure of these receptors have not been disclosed yet.

The transduction elements associated with NKp30 and NKp46 are FCeRIg and the zeta homodimer.

It was previously demonstrated that antibodies recognizing NKp30 and NKp46 could induce production of lymphokines by NK cells, and/or could induce cytotoxicity of NK cells in redirected killing assays.

The inventors demonstrate here that soluble anti NCR (NK Cell Receptor) antibodies can induce the specific proliferation of NK cells from fresh human PBMC, when used in association with cytokines. Interestingly, though CD16 shares the same transducing element (zeta homodimer and FceRIgamma), addition of soluble anti CD16 antibody did not support any specific increase of the NK cell population.

Moreover, as NKp30 and NKp46 are strictly restricted to NK cells, this demonstration gives the basis of a specific NK cell proliferation protocol.

It is thus an object of the invention to provide a pharmaceutical composition having, in particular, a stimulating effect on NK cell proliferation. It is another object of the invention to provide a method for specifically stimulating the proliferation of NK cells by using such a pharmaceutical composition.

The present invention relates also to the use of such a pharmaceutical composition in the manufacture of a drug for the prevention, palliation, and therapy of e.g., melanoma, hepatocarcinoma or lung adenocarcinoma and for anti-microbial prevention, palliation and therapy.

The pharmaceutical compositions of the invention comprise an effective amount of at least an antibody selected in the group comprising an anti-NCR antibody such as anti-NKp30 antibody or anti-NKp46 antibody, or both, or an immuno-reactive fragment thereof, and a cytokine selected in the group comprising interleukins such as IL2, IL12, IL15, IL21 or a combination thereof, in association with a pharmaceutically acceptable carrier, said antibody(ies) and cytokine(s) being administered together or separately to a subject. In a particular embodiment, the cytokine is IL2, IL15 or both. The pharmaceutical composition can comprise an expression vector encoding said cytokine. Said vector can be a viral vector and a plasmid vector. Alternatively, instead of administering said cytokine, the in vivo production of said cytokine can be induced.

In a preferred embodiment, anti-NKp30 and/or anti-NKp46 antibodies are used in admixture with IL2.

In said compositions, the anti-NKp30 antibodies are isolated antibodies or antigen binding fragments thereof which specifically bind to a polypeptide selected from the group consisting of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, or an immunogenic fragment thereof, and SEQ ID N°5.

SEQ ID N°1 relates to the human NKp30 190 aa polypeptide (about 30 kD on SDS-PAGE), which is selectively expressed by NK cells, and particularly mature NK cells ; SEQ ID N°2 relates to the extracellular region of human NKp30 receptor ; SEQ ID N°3 relates to the transmembrane region of human NKp30 receptor ; SEQ ID N°4 relates to the cytoplasmic tail of the human NKp30 receptor ; SEQ ID N°5 relates to a 15 aa immunogenic peptide derived from SEQ ID N°1.

In said compositions, "anti-NKp46 antibodies" refer to isolated antibodies respectively against NK-p46.

Preferred antibodies specifically bind to polypeptide having SEQ ID N°1.

The anti-NKp30 and/or anti-NKp46 antibodies of said compositions are advantageously monoclonal antibodies, affinity, chimerized or humanized antibodies and more preferably humanized mouse monoclonal antibodies or of human origin.

A more particularly preferred anti-NKp30 monoclonal antibody is produced by hybridoma strain I-2576.

In pharmaceutical compositions comprising immuno-reactive antibody fragments, said fragments are essentially Fab, $F(ab')_2$, Fv fragments, and CDR grafted humanized antibody fragments.

The person skilled in the art will note that humanized antibodies of the invention can be derived therefrom as desired, notably when the pharmaceutical compositions according to the invention are intended to be administered to a human person. By « antibody immuno-reactive fragments », it is herein notably meant any antibody fragment comprising the antigen binding-site. Such fragments thus include $F(ab')_2$ fragments obtained either by enzymatic digestion of said antibody by proteolytic enzymes such as pepsin or papaïn and Fab fragments derived thereof by reduction of sulfhydryl groups located in the hinge regions, as known by any skilled person. Immunoreactive fragments can also comprise recombinant single chain or dimeric polypeptides whose sequence comprises the CDR regions of the antibody of interest. Isolated CDR regions themselves are also contemplated within the definition of the isolated immuno-reactive fragments.

Said pharmaceutical compositions can be administered by various routes, including intradermal, intramuscular, intraperitoneal, intravenous, or subcutaneous injection, intranasal route and the chirurgical route.

Depending on the desired administration route, the galenic forms will be, for example, tablet, powder, pastes, patches, granules, microgranules, nanoparticules, colloid solution, aqueous solution, injectable solutions, sprays and liposomes. The galenic form may also correspond to slow and/or controlled release forms.

By « pharmaceutically acceptable vehicle » comprised in the pharmaceutical compositions of the invention it is meant herein a vehicle whose solubility and/or chemical and/or galenic properties are adapted to the desired administration route and the ailed efficiency level. Such vehicles may include saline or dextrose solutions. The pharmaceutical composition according to the invention may further comprise any appropriate buffer and/or stabilizing compound.

Generally speaking, the pharmaceutical compositions of the invention are useful in the pathologies susceptible to be controlled by NK cells.
Numerous cancer have been shown to be susceptible to NK cell lysis, i.e. melanoma, Chronic Myeloid Leukemia, Acute Myeloid Leukemia, Lymphomas, Multiple Myeloma, hepatocarcinoma, lung adenocarcinoma, Neuroblastoma... Virally infected cells are also susceptible to NK cell lysis such as CMV, EBV, HIV, HCV etc.

The pharmaceutical compositions of the inventions are particularly useful for anti-tumoral prevention, palliation, therapy e.g., of melanoma, hepatocarcinoma or lung adenocarcinoma and for anti-microbial prevention, palliation and therapy.

The dosage will be chosen depending on the condition of the patient to be treated.

An effective dose typically ranges from 1 ng to 100 mg/kg (body weight) of anti-NCR antibodies, and typically lower than 1 million units/square meters/day of cytokine(s), when the pharmaceutical composition of the invention is used for daily subcutaneous injection. In fact, the amount of anti-NCR antibody to be used in such an in vivo pharmaceutical composition of the invention to obtain a specific proliferation of NK cells will notably depend on the particular antibody or antibodies used (affinity, chimerized or humanized antibody). The antibody should preferably be used to obtain an effective concentration for stimulation, without inducing a depletion of the NK cells or toxicity.

Advantageously, said interleukine is IL-2 and is injected subcutaneously at daily doses below 1 million units/m^2 for 5 to 10 days.

The invention also relates to a method for stimulating the proliferation of NK cells which comprises contacting NK cells with an effective amount of a pharmaceutical composition as above defined.

Advantageously, the method of the invention comprises one or several injections of an effective amount of at least an antibody selected in the group comprising an anti-NCR antibody such as anti-NKp30 antibody or anti-NKp46 antibody, or both, or an immuno-reactive fragment thereof, and, repeated injections of a cytokine selected in the group comprising interleukins such as IL2 (Research Diagnostics, NJ, RDI-202), IL12 (Research Diagnostics, NJ, DI-212), IL15 (Research Diagnostics, NJ, RDI-215), IL21 (Asano et al, FEBS Lett. 2002;528:70-6) or a combination thereof, during 5-10 days, said cytokine(s) being

first injected on the same day as the first injection of antibodies. Preferably, the cytokine is IL2, IL15 or both.

Said method preferably comprises one or two injections/day of cytokine(s) by subcutaneous route.

The invention also relates to the use of said pharmaceutical composition in the manufacture of a drug for the antitumoral prevention, palliation, and therapy of e.g., melanoma, Chronic Myeloid Leukemia, Acute Myeloid Leukemia, Lymphomas, Multiple Myeloma, hepatocarcinoma, lung adenocarcinoma, Neuroblastoma and for microbial prevention, palliation an therapy.

These and other features and advantages of the invention will be further apparent from the following examples. These examples are given for illustrative purposes only, and are in no way intended to restrict the scope of the present invention. Alternatives embodiments intended by any skilled person are encompassed by the present invention.

Description of the drawings

In these examples, reference is made to figures 1 to 6.

Figure 1: Peripheral blood mononuclear cells (PBMC) from one healthy donor was cultivated with indicated antibodies (AZ20= anti NKp30, Bab281= anti NKp46), at 10 or 30 μg/ml in the presence of 50 units/ml IL2 until day 6, and either 50 units/ml (black bars) or 400 units/ml green bars) from day 6 to day 10. % of NK cells was determined by flow cytometry at day 10.

Figure 2: Relative fold increase of NK (%NK cell at indicated day divided by %NK cells at day 0) from total unfractioned PBMC from 4 healthy donors with

10µg/ml indicated antibodies at start and 50 units/ml IL2 along culture. Mean of relative fold increase, +/- standard deviations are represented.

Figure 3 : Carboxyfluorescein succinimidyl ester (CFSE) labelling (FL1, log scale, X axis) of NK cells (gated on CD56+/CD3- cells after 6 days of culture with the indicated treatment.

Figure 4. AZ20 combined with IL-2 or IL-15 induce NK cells expansion. Freshly isolated PBMC were cultured under different conditions of interleukins (from day 0 to day 6 : concentration is the bottom one; from day 6 to day 13 : concentration of interleukin is the upper one) and with either an anti-CD56 mAb (N901, IgG1, 10µg/ml) or an anti-NKp30 mAb (AZ20, IgG1, 10µg/ml). At day 13 cells were collected and analyzed by flow cytometry for the % of NK cells defined as CD56+CD3- lymphocytes.

Figure 5 : Freshly isolated PBMC from 3 donors (A,B,C) were cultured with the indicated amount of AZ20 in RPMI 1640 10%FCS containing IL-2 (50u/ml from day 0 to 6 and 400u/ml from day 6) and IL-15 (10ng/ml). At day 13 cells were collected; viability and count were assessed by trypan blue and % $CD56^{+}CD3^{-}$ lymphocytes by flow cytometry.

Figure 6 : CD25 induction of NK cells obtained after 6 days of stimulation of PBMC of two healthy donors (see material and methods), with indicated stimulus (IL2 (50 U/ml), mAbs 10 µg/ml).

1. Materials and methods

Materials:

. Blood:

- For the first experiments, peripheral blood (5 to 10 x 7ml EDTA-tubes, Becton Dickinson #367655) was collected from healthy volunteers (Lab. Hématologie, La Conception) and processed within two hours.
- For further experiments, peripheral blood samples were provided by Etablissement Français du Sang (EFS) and processed within 24 hours (the blood is collected in bag containing 63ml of anticoagulant CPD for the collect of 450ml± 10% blood; Baxter #R8443).

. PBMC and Primary cell culture:

- 50ml polypropylene conical tubes (Falcon, #35 2070).
- 96 well plate U form (Falcon, #35 7525)
- RPMI 1640 medium (Invitrogen, #31870074)
- Fetal calf Serum (Invitrogen, #10270-106, Lot #40A0285K) heat inactivated
- Penicillin-Streptomycin (5000u/ml, Invitrogen, # 15070071)
- Sodium Pyruvate (100mM, Invitrogen, # 11360088)
- L-Glutamine 200mM (100X, Invitrogen, #25030123)
- Ficoll-Paque™ PLUS (Amersham Pharmacia Biotech, #17-1440-03)
- Trypan Blue 0.4% (Invitrogen, #15250061)
- D-PBS (1X) (Invitrogen, # 14190169)
- Hemacytometer (Neubauer)
- Human recombinant IL-15 (25µg, R&D, # 219-IL-025). Stock solution of IL-15 (10µg/ml) was prepared in PBS/BSA 0.1%, aliquoted and stored at -20°C.
- Human recombinant IL-2 (Proleukin, $18x10^6$ IU, batch A199606/2, Chiron). Stock solutions of IL-2 ($2x10^6$ and $2x10^5$u/ml) were prepared in PBS/BSA 0.2% aliquoted and stored at -20°C.

- Monoclonal antibodies :

- 3G8 (anti-CD16), 5mg/ml, Beckman Coulter Immunotech.

- N901 (anti-CD56), 5mg/ml, Beckman Coulter Immunotech.

- Bab281 (anti-NKp46), 2,8mg/ml (mAb purified on protein A Sepharose® from mice ascites)

- AZ20 (anti-NKp30), 1mg/ml and 1.2mg/ml (mAb purified on protein A sepharose from mice ascites).

. Cell division analysis (CFSE labelling):

5-(and 6)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE) **mixed isomers** (CFSE, 25mg ; Molecular Probes, C-1157)

DMSO hybri-Max® (Sigma #D 2650)

Stock solution of CFSE (10mM) in DMSO was prepared, aliquoted and stored at -20°C as described in the technical data sheet provided by Molecular Probes.

. Staining:

- 96 well plate U form (Greiner #650 180)

- 1.2ml micro titer tubes (QSP, #845-F)

- 5 ml tubes (12x75 PRO, CML, #TH5-12PRO)

- Staining Buffer: PBS/0.2%BSA/0.02% Sodium Azide (D-PBS (10X), Invitrogen #14200083; Albumin Bovine, Fraction V, Invitrogen #; Sodium azide, Prolabo #27 967.150)

- Mouse Serum NMRI (Janvier)

- Commercialy available Ab used in this study (Table 1)

. Flow cytometry:

Samples were run on a XL/MCL cytometer (Beckman Coulter). Acquisition and analysis were performed with EXPO™ 32 v1.2 software (Beckman Coulter).

Methods:

. Preparation of PBMC:

Blood samples were diluted volume/volume with RPMI and processed using a classical ficoll procedure.

PBMC were collected in 50ml conical tubes, washed 4 times with RPMI, 2% FCS, counted with trypan blue. Cells were resuspended at 2*10EE6 cells per ml in complete medium (RPMI 1640, FCS 10%, PS (50u/ml), Glu 2mM, Na. Pyr. 1mM) for the initiation of cell culture, or 10EE7 cells/ml in staining buffer (PBS, 0.2% BSA, 0.02% Sodium Azide) for flow cytometry experiments.

. CFSE labelling:

- PBMC (10^7cells/ml) were incubated 10 to 25 minutes at 37°C (Water Bath) in RPMI/FCS2% containing CFSE (5 to 10 µM).
- Cells were washed 3 times (10 min, 1200RPM) with large volumes of cold (4°C) RPMI/FCS 2%.
- PBMC were resuspended in complete medium ($2x10^6$cells/ml) and were ready for cell culture.

For each of the subsequent technique, inventors recommend the following steps.

. Primary cell culture:

Day 0

- Resuspend PBMC ($2x10^6$/ml) in complete medium (RPMI 1640, FCS 10%, PS (50u/ml), Glu 2mM, Na. Pyr. 1mM).
- Prepare 2X interleukin stocks (IL-2, IL-15 and IL-2+IL-15) with complete medium.

Depending of the experiment, IL-2 was used at 50 or 400u/ml final. IL-15 was always used at 10ng/ml final.

- Prepare 4X antibody stocks with complete medium.

- Set up the culture: 50µl 4X mAb + 100µl 2X interleukine + 50µl PBMC (10^5cells/well)

fill up to 200µl with complete medium.

Day 3:

- Change medium: remove 100µl and add 100µl complete medium containing 1X interleukin.

Day 6:

- Change medium: remove 100µl and add 100µl complete medium containing either 50u/ml IL-2 (± IL-15 10ng/ml) or 400u/ml IL-2 (± IL-15 10ng/ml).

Day 9:

- Split cells 1 /2 and add medium (day 6)

Day 13, 16 and 20:

- same as day 6 or day 9 depending of the cell growth.

. Staining:

- Volumes, dilutions and Ab concentrations used in this study are indicated in Table 1.

- For staining of cultured PBMC, 1 or 2 wells might be used for 1 point of staining. Control samples were prepared with interleukin stimulated cells.

- % NK cells (defined as $CD56^+CD3^-$ cells) were checked at day 0, day 6, day 13, day 16 and day 20 and for some experiments at day 3, day 9 and day 35.

- Characterization of the NK cell and T lymphocyte compartments at day 0 and day 17 (for some experiments) with antibodies listed in Table 1

Distribute mAb and adjust volume to 50µl with staining buffer.

Add 50 µl of cell suspension.

Incubate 30 mns on ice.

Wash two times with staining buffer.

Resuspend cells in 150µl of staining buffer and transfer to RT15 tubes containing 150µl of staining buffer.

Keep refrigerated until acquisition on flow cytometer.

. Cytometry:

Acquisition:

- Run the isotype control mix in "set up mode" and set up: FSC, SSC, Threshold, FL1, FL2, FL3 and FL4 parameters:

. Analysis was focused on lymphocytes identified by their FSC and SSC features (FSC, linear, Gain: 2, Volts: 400 and SSC, linear, Gain: 20, Volts: 400; Threshold: FSC, 150); the volts of these parameters might slightly differ analyse each experiment of this study (the FSC and SSC of cultured cells are usually higher than those of freshly isolated cells).

. Draw the lymphocyte gate= Ly (acquire at least 10 000 events in Ly but all the events are collected)

. Set up the volts for each fluorescent probe used in the experiment (aproximatively, FL1=800; FL2=800; FL3=950 and FL4=1000); they might slightly differ between each experiment).

- Set up the compensations using single staining sample (mAbs used in the experiment or anti-CD8):

. first, run FL1-mAb sample and set up FL2-FL1 (=15-20) in order to have the same FL2-MFI for the FL1-negative and the FL1-positive cells and all the FL2-negative cells in the first decade (< 0.5% in the FL2 histogram and in FL1/FL2 dot plot); then, set up FL3-FL1 and FL4-FL1 as just described for FL2-FL1. Write the values and clear the compensations.

- Repeat this step for each fluorescent probe.

- Copy all the values to the compensation matrix.

- Acquire the isotypic control sample and then, all the samples prepared for the experiment (write the lmd number corresponding to the acquired sample on the "96 well table").
- Each sample (lmd) is recorded and then transfert in a folder called: year, month, day (for example: 20020126). This folder is located in the HC/PA folder.

- Acquisition of CFSE samples:

. FL1 compensations must be done using CFSE labelled cells only.

. First set up the volts for FL1, FL2,... with the isotypic control sample; then, run the CFSE sample. The labelling is good when all the cells are positive for CFSE, the staining homogeneous and the pic channel located in the middle of the last decade of the FL1 histogram (without lowering the FL1 volts).

. Set up all the compensations (they are usually higher than those obtained with FL1-mAb stained cells).

Analysis:

- Analysis was focused on lymphocytes identified by their FSC and SSC features (dot plot FSC/SSC). Draw the lymphocyte gate (Ly).

Quadrant regions (for dot plot) and marker regions (for histogram) were set with isotypic control samples (for all the fluorescences: % FLX^+ cells < 0.5%)

- Analysis of the T cell or NK cell compartments:

T cells= $CD3^+$ lymphocytes were defined as the positive cells of the anti-CD3 staining histogram gated on Ly.

NK cells= $CD3^-CD56^+$ lymphocytes corresponds to the $CD3^-CD56^+$ gate in the CD3/CD56 dot plot (upper left part of the quadrant).

CFSE staining, CD25 expression (%), NKR expression (%) and CD56 density (MFI) were analysed.

. Cell count and freezing:

Some cultures were checked for cell numbers and cell viability (Trypan blue exclusion) and then frozen at the end of the experiment (day 20 or 35).

Voir les commentaires à donner pour les colonnes 2, 5 et 6

Table 1. Antibodies and reagents for cell cytometry.

Specificity	Clone	Isotype	Cond.	Origin	Cat.#	Vol./Conc./dilution
CD3	UCHT1	mIgG1	FITC	BC Iot	IM1281	5µl
CD3	UCHT1	mIgG1	PE	BC Iot	IM1282	5µl
CD3	UCHT1	mIgG1	PC5	BC Iot	IM2635	5µl
CD3	UCHT1	mIgG1	ECD	BC Iot	IM2705	5µl
CD8	B9.11	mIgG1	FITC	BC Iot	IM0451	5µl
CD8	B9.11	mIgG1	PE	BC Iot	IM0452	5µl
CD8	B9.11	mIgG1	PC5	BC Iot	IM2638	5µl
CD8	SFCI21ThyD3	mIgG1	ECD	BC	6607011	5µl
CD16	3G8	mIgG1	purified	BC Iot	813	
CD16	3G8	mIgG1	PE	BC Iot	IM1238	5µl
CD16	3G8	mIgG1	FITC	BC Iot	IM0814	5µl
CD25	B1.49.9	mIgG2a	FITC	BC Iot	IM0478	10µl
CD25	B1.49.9	mIgG2a	PC5	BC Iot	IM2646	5µl
CD25	M-A251	mIgG1,k	PE	BD	555432	5µl
CD27	1A4-CD27	mIgG1,k	PE	BC Iot	2578	5µl
CD45	IMMU 19.2	mIgG1	PC5	BC Iot	IM2652	5µl
CD54	84H10	mIgG1	PE	BC Iot	IM1239	5µl
CD56	N901(NKH-1)	mIgG1	purified	BC Iot	6602705	
CD56	N901 (NKH-1)	mIgG1	PE	BC Iot	IM2073	5µl
CD56	N901 (NKH-1)	mIgG1	PC5	BC Iot	IM2654	3µl
CD57	NC1	mIgM	PE	BC Iot	IM2377	5µl
CD62L	DREG56	mIgG1	PE	BC Iot	IM2214	5µl
CD69	TP1.55.3	mIgG2b	PE	BC Iot	IM1943	4µl
CD94	HP-3B1	mIgG2a	PE	BC Iot	IM2276	5µl
CD122	CF1	mIgG1	PE	BC Iot	IM1978	5µl

CD158a	EB6	mIgG1	PE	BC Iot	IM2277	10µl
CD158b	GL183	mIgG1	PE	BC Iot	IM2278	5µl
CD158e1/e2	Z27	mIgG1	PE	BC Iot	IM3292	5µl
CD158i	FES172	mIgG2a	PE	BC Iot	IM3337	5µl
CD158k	Q66	mIgM	ascite	BC Iot		1: 2000
CD159a	Z199	mIgG2b	PE	BC Iot	IM3291	5µl
CD161	191B8	mIgG2a	PE	BC Iot	IM3450	5µl
CD162R	5H10	mIgM	biotin	IP (AT,HC)		1µg/ml
CD244	C1.7.1	mIgG1	PE	BC Iot	IM1608	5µl

Isotype control	679.1Mc7	mIgG1	purified	BC Iot	IM0571	10µl
Isotype control	679.1Mc7	mIgG1	ECD	BC Iot	IM2714	5µl
Isotype control	679.1Mc7	mIgG1	FITC	BC Iot	IM0639	5µl
Isotype control	679.1Mc7	mIgG1	PE	BC Iot	IM0670	5µl
Isotype control	679.1Mc7	mIgG1	PC5	BC Iot	IM2663	3µl
Isotype control	U7.27	mIgG2a	PE	BC Iot	IM0671	5µl
Isotype control	MOPC-195	mIgG2b	RD1	BC Iot	6603038	5µl
Isotype control	GC323	mIgM	purified	BC Iot	IM1268	10µl
Isotype control	GC323	mIgM	RD1	BC Iot	6602940	5µl

<u>EXAMPLE 1.</u> ANTI NCR ANTIBODIES + IL2 CAN PROMOTE SPECIFIC CELL PROLIFERATION OF NK CELLS.

- Relative amplification of NK cells after stimulation with anti NCR antibodies and IL2.

PBMC from one donor has been isolated and tested for their in vitro response to combination of IL2 with either CD16, NKp30, NKp46 or CD56 mAbs. Cells were treated as described in material and methods, in the presence of saturating amount of antibodies.

Cells were monitored by flow cytometry and relative percentage of CD56+/CD3- (NK cells) was determined.

The results are presented in figure 1.

For this donor, there was an enrichment in NK cells in the culture at day 10 in the presence of anti NCR antibodies, whereas CD16 or CD56 induced no significant enrichment relative to IL2 alone.

To evaluate if this enrichment is donor related or not, PBMC have been isolated from 4 healthy volunteers and tested for their in vitro response to combination of IL2 + monoclonal antibodies against NCR.
Cells were treated as described in material and methods and put in the presence of saturating amounts (10 µg/ml) of either no antibodies,anti-NKp30, anti-NKp46, combination of NKp30 and NKp46, anti-CD56 monoclonal antibodies.
Cells were monitored by flow cytometry and relative percentage of CD56+/CD3- (NK cells) was determined.

The results are presented in figure 2.

For the four healthy donors tested, there was a selective enrichment in NK cells. The enrichment is slightly better when anti NKp30 is used as compared to anti NKp46. The combination of the two antibodies gives the best enrichment.

The conclusion of these two studies is that the combination of anti NCR antibodies, with low dosage of IL2 (50 units/ml), induces a selective enrichment of NK cells.

To evaluate if this expansion is due to effective proliferation of NK cells or to selective death of the other cells present in the culture, PBMC were stained with CFSE, then washed to get rid of excess dye, at the initiation of the culture (see material and methods). CFSE is a stable fluorescent label that attach covalently to the cells. When the cells divide, about half of the initial dye content is present on the two daughter cells. If cells divide again, $1/4^{th}$ of the initial dye content is present on the 4 daughter cells etc. Labelled cells were put in culture and

stimulated by anti NCR antibodies and IL2 as above. Dye content of the cells is monitored by flow cytometry.

The result obtained on one representative donor is given in figure 3.

NKp30 or NKp46 + IL2 co-treatment induces a better proliferation of NK cells than IL2 alone or IL2 + irrelevant mAb (CD56) as indicated by the numbers of cells remaining with fluorescence intensity equivalent to resting cells (no division) : 50 and 40 % for IL2 and IL2 + CD56 respectively, and 5 and 11 % for NKp30 + IL2 and NKp46 + IL2 respectively.

The best proliferation was obtained for NKp30 where more than 80 % of the cells in the culture at day 6 underwent more than 5 divisions.

To conclude, anti NCR (NKp30, NKp46 or both) + IL2 co-treatment induce selective proliferation of NK cells from PBMC in vitro.

EXAMPLE 2. ANTI NCR + IL15 ALSO INDUCES THE SPECIFIC EXPANSION OF NK CELLS.

The presence of a cytokine is crucial to sustain the expansion of the cells, after stimulation with the antibody. Experiments were carried out for testing if IL15 could also sustain the expansion of the cells on one donor.

Cells were stimulated with anti NKp30, and cultured in the presence of IL2, IL15 or both.

The results are presented in figure 4.

On this donor, IL15 was able to sustain the proliferation of NK cells.

We checked also that the combination of IL2 and IL15 also sustain the proliferation of NK cells. In other experiments on other donors, it was observed that the combination of IL2 and IL15 can be slightly better than the two cytokines alone.

EXAMPLE 3. TITRATION CURVE OF ANTI NCR ANTIBODIES FOR INDUCTION OF PROLIFERATION.

To evaluate the amount of antibody necessary to obtain a proliferation, a titration curve has been established with 3 independent donors with anti NKp30 antibody. The results are shown in figure 5. This experiment shows that the effect of the antibody is saturable with a plateau effect at about 1 µg/ml. The dose to obtain 50 % of maximum effect is below 0.1 µg/ml in this experiment.

It should be noted that the characteristics of the curve may depend on the particular antibody used, and particularly of its affinity. The use of humanized anti NCR antibodies may also display a different titration curve.

EXAMPLE 4. CONDITIONS OF USE OF ANTI NCR ANTIBODIES + IL2 IN VIVO.

The anti-NCR antibody or antibodies were tested first in vitro, and then in a relevant animal model.

It should be noted that anti NCR + IL2 in vitro induces CD25 (Fig 6), and thus the high affinity receptor for IL2 on most NK cells. In vitro, low doses such as 50 units/ml are sufficient to sustain the proliferation of NK cells. Thus, it can be anticipated that low dose IL2 (typically lower than 1 million units/square meters/day for daily subcutaneous injection) will be sufficient to sustain proliferation. In vitro, CD25 down regulated after 9-10 days, so that it is anticipated that the length of the low dose IL2 treatment will be up to 10 days.

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DELIVER THE ATTACHED FIFLE/DOCUMENT TO THE TC SCANNING CENTER

CONTRACTOR: THE ATTACHED FILE/DOCUMENT MUST BE INDEXED AND SCANNED INTO IFW WITHIN **8** WORK HOURS; UPLOADING OF THE SCANNED IMAGES SHOULD OCCUR NO LATER THAN **16** WORK HOURS FOLLOWING RECEIPT OF THIS REQUEST

AFTER SCANNING, ORIGINAL DOCUMENTS SHOULD BE BOXED IN ACCORDANCE WITH INSTRUCTIONS